IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	A.S. Hoffman et al.	Attorney Docket No.:	UWOTL118949
Application No.:	09/226,044	Group Art Unit:	1615
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Title: ENHANCED TRANSPORT USING MEMBRANE DISRUPTIVE AGENTS

DECLARATION OF PATRICK STAYTON UNDER 37 C.F.R. § 1.132

Seattle, Washington 98101

March 11, 2003

TO THE COMMISSIONER FOR PATENTS:

I, Patrick Stayton, declare as follows:

1. I am a Professor in the Department of Bioengineering at the University of Washington, Seattle, Washington, and have been since 1992. I received a B.S. in biology from Illinois State University in 1984 and a Ph.D. in biochemistry from the University of Illinois in 1989. I have conducted research and published scientific papers related to stimuli-responsive polymers.

2. I directed and reviewed the results of the syntheses of poly(2-methylacrylic acid), poly(2-ethylacrylic acid), and poly(2-propylacrylic acid) described below.

Monomer and Polymer Synthesis. All chemicals and solvents were purchased from Sigma (MI, USA) and were of analytical grade unless otherwise stated. Ethyl and propyl acrylic acid monomers were prepared according to the procedure outlined by (Ferrito and Tirrell, 1992). Methylacrylic acid monomer was purchased from Lancaster (NH, USA). The structures of the monomers were verified by NMR. Poly(2-methylacrylic acid) (PMAA), poly(2-ethylacrylic acid) (PEAA), and poly(2-propylacrylic acid) (PPAA) were prepared as described previously (Murthy et al., 1999). Briefly, polymers were synthesized by free radical polymerization using 2,2'-azobisisobutyronitrile (AIBN) as the initiator at 60°C for 48-72 h. The polymers were

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dissolved in methanol and fractionated and purified by ether precipitation. The molecular weights of the PMAA (52 kD), PEAA (43 kD), and PPAA (44 kD) fractions used in this study were determined by aqueous gel permeation chromatography (GPC) using PEG standards.

The structures of poly(2-methylacrylic acid), poly(2-ethylacrylic acid), and poly(2-propylacrylic acid) are illustrated in FIGURE 1 attached.

3. I directed and reviewed the results of the hemolysis assay described below.

Hemolysis Assay. The pH-dependent activity of the polymers was tested utilizing a red blood cell (RBC) hemolysis assay as described previously (Lackey et al., 1999; Murthy et al., 1999). The release of hemoglobin from the RBCs was used as a measure of the membrane-disruptive activity of these polymers. Fresh human RBCs were isolated from whole blood by centrifugation and washed three times with 0.15 M NaCl. The RBCs were resuspended in either 100 mM phosphate or 75 mM citrate-phosphate buffer at the desired pH, then diluted in the appropriate buffer to obtain approximately 10^8 RBCs/ml. Polymer (PMAA, PEAA, or PPAA) and buffer were added to the RBC suspension, and the samples were incubated in a 37°C water bath for 1 h. In experiments testing the effects of concentration and pH on polymer-mediated hemolysis, the polymers were used at 5 – 50 μg/ml and 10 μg/ml, respectively. The samples were then centrifuged for 4 min to pellet any remaining intact RBCs. The absorbance of the supernatant from each sample was measured at 541 nm using a spectrophotometer. Each test was performed in triplicate. Hemolysis levels were normalized to both negative and positive controls. In controls, RBCs were incubated as above in either buffer alone or buffer containing 1 mg/ml dextran (negative control) or in distilled water or buffer containing 10 mg/ml Triton-X 100 (positive control).

The hemolysis results are illustrated in FIGURE 2 attached.

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4. I directed and reviewed the results of the cell culture and transfection experiments described below.

Cell Culture and Transfection. NIH3T3 (Swiss mouse embryonal) cells (obtained from ATCC) were cultured in DMEM supplemented with 10% FBS. All cell cultures were maintained in a 37°C incubator with 5% (v/v) CO_2. In experiments testing the ability of polymers to facilitate transfection, NIH3T3 cells were seeded onto twelve-well plates and incubated at 37°C for 24 hr in DOTAP lipoplexes were prepared following a specific mixing protocol. DOTAP was added to the plasmid DNA (pCMVβ) and complex formation allowed to proceed for 30 min. The polymer (PMAA, PEAA, or PPAA) was then added to the DOTAP/DNA mixture for an additional 30 minutes. For control mixtures, HEPES-Buffered Saline (HBS) was added to DOTAP/DNA formulations instead of polymer. All formulations contained 1.6 μg/ml DNA. For formulations containing polymer, the DOTAP concentration was set to 10 μg/ml and polymer concentrations were adjusted to reach the specified theoretical particle charge ratio. The theoretical particle charge ratio was determined as the molar ratio of positively charged amines in DOTAP to the cumulative total of all negatively charged phosphate and carboxyl moieties in the DNA and polymer, respectively. For control formulations, DOTAP concentrations were varied to achieve the proper particle charge ratio. Cells were transfected in serum-free DMEM with these DOTAP formulations for 4 h at 37°C. All formulations were tested in triplicate wells. After 4 h, the transfection particles were removed and replaced with DMEM supplemented with 10% FBS. The cells were then left to incubate at 37°C for 48 h to allow for protein expression. β-Galactosidase gene expression was evaluated by utilizing the β–Galactosidase Enzyme Assay System (Promega, WI, USA). o-Nitrophenyl- β–D-galactopyranoside (ONPG) was added to cell extracts recovered upon lysing the cells, and the amount of β-galactosidase activity was measured spectrophotometrically at

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415 nm. Concurrently, the total cellular protein content in each of the extracts was determined using the BCA reagent (Pierce, IL, USA). Protein concentrations were calculated from absorbance measurements at 570 nm. Normalized β-galactosidase activity was determined from the β-galactosidase activities normalized to total protein content values.

The results of these experiments are illustrated in FIGURE 3 attached.

5. Although poly(2-methylacrylic acid), poly(2-ethylacrylic acid), and poly(2-propylacrylic acid) can be considered to be homologs, these polymers have dramatically different properties.

Referring to FIGURE 2, while PEAA and PPAA show hemolytic activity within the range for endosomal acidification (e.g., about pH 5 to about pH 7.4), PMAA does not. PPAA shows activity at a higher pH range compared to PEAA activity. Like PMAA, poly(acrylic acid), the parent polymer, also demonstrates no hemolytic activity in this pH range.

Referring to FIGURE 3, for example at charge ratio 1, PPAA, PEAA, and PMAA, all exhibit a positive effect on transfection as measured by enzymatic activity compared to control. PPAA demonstrated the greatest effect on activity and had significantly greater activity than either PEAA or PMAA.

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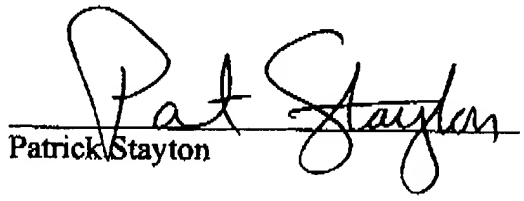
6. All statements made herein and of my own knowledge are true; and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge and belief that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,



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